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REVIEW ARTICLE

Endocytosis: a review of mechanisms and plasma membrane dynamics

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Introduction

Endocytosis is the process of internalization of extracellular material within an invagination of the plasmalemma. Ultimately, this invagination is thought to give rise to an independent cytoplasmic vesicle. This vesicle is given a more specific name (e.g. phagosome, pinosome, coated vesicle, primary food vacuole) depending on the cell type being studied and the mode of endocytosis involved. Historically, the multitude of morphological varieties of endocytosis has led to a plethora of confusing terminology (e.g. cytosis, endocytosis, intracytosis). However, it was de Duve in 1963 who used the term 'endocytosis' to encompass all variant forms of this basic cellular transport mechanism.

Classically, endocytosis has been divided into two types: phagocytosis, or eating, and pinocytosis, or drinking. The term phagocytosis is used to describe the internalization of particulate matter visible by light microscopy and, hence, larger than a few tenths of a μm in diameter. The term pinocytosis is used to describe the uptake of all smaller substrates, ranging from insoluble particles to low molecular weight solutes and to the fluid itself. Pinocytosis can be subdivided further, based on whether the substance enters the cell in the fluid content (fluid-phase pinocytosis) or bound to the vesicle membrane (adsorptive pinocytosis). Moreover, adsorptive pinocytosis can be subdivided further into endocytosis of markers which bind in a non-specific manner to the cell surface (e.g. cationic ferritin binding to anionic sites on the plasmalemma), and endocytosis of ligands mediated via binding to specific receptors on the cell membrane. The latter mechanism has been called receptor-mediated endocytosis (pinocytosis). Molecules known to be taken up by this receptor-mediated mechanism include lysosomal enzymes (Neufeld *et al.*, 1977), polypeptide hormones [such as epidermal growth factor (Carpenter & Cohen, 1976) and insulin (Maxfield *et al.*, 1978)], thyroid hormones (Cheng *et al.*, 1980), plasma transport proteins [such as low density lipoprotein (Brown & Goldstein, 1979)], asialoglycoproteins (Ashwell & Morell, 1974) and α_2-macroglobulin (Willingham *et al.*, 1979).

The purpose of this review is to summarize recent findings relating to mechanisms of both phagocytosis and pinocytosis. With regard to phagocytosis, we focus attention on questions concerning the nature of particle interaction with the cell membrane, as well as the uptake mechanism itself. With regard to pinocytosis, we review methods of quantification as well as describe what is known of the uptake and recycling of pinocytosed membrane and solute. A scheme of the intracellular pathways involved is provided in Fig. 1. Though both fluid-phase and receptor-mediated mechanisms are discussed, we emphasize the former since the latter has received extensive review recently (Goldstein *et al.*, 1979; Ockleford & Whyte, 1980; Pearse, 1980; Pastan & Willingham, 1981).

Phagocytosis

Experimentally, phagocytosis has been divided into two steps: (*a*) attachment of the particle to the cell surface and (*b*) internalization of the particle. Each of these processes in its turn depends upon a number of events of which Stossel (1976) has defined seven: (1) certain particle surfaces elicit recognition by the phagocyte; (2) the phagocyte receives the message of recognition; (3) the phagocyte transmits the message to its cytoplasm, arousing effector mechanisms; (4) the plasma membrane adheres strongly to the particle; (5) pseudopods assemble and (6) move around the particle; and (7) the tips of the pseudopods fuse at the distal side of the particle. It is through investigation of such discrete steps that will come an understanding of the phagocytic process.

Abbreviation used: GERL, Golgi apparatus–endoplasmic reticulum–lysosomes.

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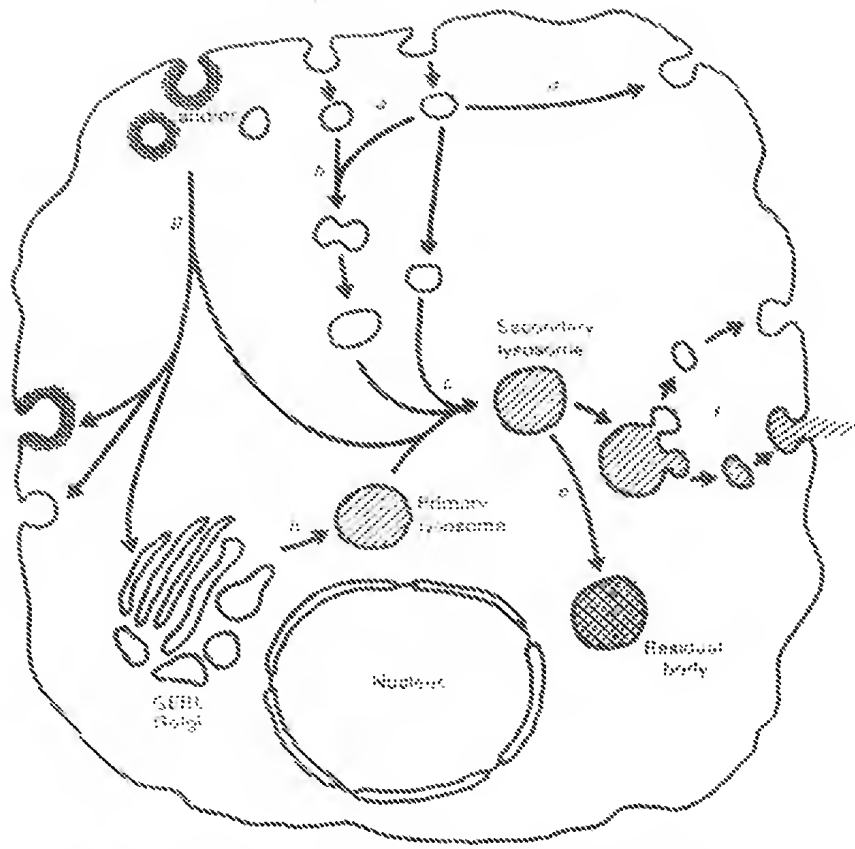

Fig. 1. *Schematic representation of pinocytotic vesicle pathways*
This diagram illustrates known and suggested routes involved in the dynamic flow of pinocytotic vesicle membrane and contents in a generalized eukaryotic cell. For sake of clarity, pathways are classified into two types: fluid-phase pinocytosis is shown as occurring via uncoated vesicles (steps *a–f*) and adsorptive (receptor-mediated) pinocytosis as occurring via coated pits and then either coated or uncoated vesicles (steps *g–h*; see below). (*a*) Formation of pinosomes from uncoated pits (invagination). (*b*) Fusion of pinosomes with each other. (*c*) Fusion of pinosome with primary lysosome to form secondary lysosome. (*d*) 'Short-circuit' pathway resulting from fusion of pinosome with plasmalemma and exocytosis. (*e*) Secondary lysosome gives rise to residual body. (*f*) Vesicles arising from secondary lysosomes fuse with plasmalemma, resulting in exocytosis (these vesicles may or may not contain lysosomal enzymes). (*g*) Coated pit pinches off from plasmalemma, giving rise to either coated vesicle or to uncoated vesicle (i.e. 'receptosome'). Either structure, in turn, can go through a fusion step (analogous to *b*; not shown), fuse with plasmalemma, fuse with Golgi/GERL, or fuse with primary lysosomes. (*h*) Vesicles arising from Golgi/GERL fuse with primary lysosomes.

Quantification of phagocytosis

Phagocytosis can be studied by using cells either in suspension or monolayer (for review, see Kavet & Brain, 1980). The major disadvantage in suspension assays is the difficulty in rapidly and cleanly separating the cells from their phagocytic substrate, though there are solutions to this problem (Kavet & Brain, 1977). On the other hand, monolayers are easily and rapidly washed free of non-associated particles and for this reason are generally considered superior for studies of the kinetics of particle uptake.

Since endocytic processes transfer matter from extracellular to intracellular compartments, one may quantify particle ingestion *in vitro* by measuring the disappearance of extracellular particles and/or their intracellular accumulation. Typical of the former approach was the work of Cohn & Morse (1959) and Sastry & Hokin (1966). This approach, however, is plagued with both practical and theoretical problems due to the need for generating measurable changes in extracellular particle concentration. Measuring intracellular accumulation, though more sensitive, is by no means simple and problem-free.

Quantification can be achieved by either direct microscopic enumeration [light or electron (Sbarra & Karnovsky, 1959; Rabinovitch, 1967)], spectrophotometric analysis (Roberts & Quastel, 1963; Stossel *et al.*, 1972) or radioassay (Brzuchowska, 1966; Carpenter & Barsales, 1967; Michell *et al.*, 1969; Al-Ibrahim *et al.*, 1976). Unfortunately, such assay procedures do not always include a technique to discriminate adequately between particles internalized and those merely adsorbed to the cell surface. In order to do so, a variety of approaches have been tried. Rabinovitch (1967) discriminated between attached and ingested red blood cells by their different hues; Axline & Reaven (1974) considered latex spheres ingested if they fell into the same focal plane as the structural elements of the cytoplasm, and Kavet & Brain (1977) claimed that it was possible to rotate individual cells by 180° and thereby expose adherent particles. Mantovani *et al.* (1972) lysed only surface-adherent sheep red blood cells by hypotonic shock, while Dilworth *et al.* (1975), after incubation of a polymorphonuclear leukocyte monolayer with gonococci, exposed the cell layer to fluorescein-tagged rabbit anti-gonococcal serum at 4°C. Gonococcal-specific antibody attached only to the externally adherent bacteria. Using [^{3}H]thymidine-labelled staphylococci, Peterson *et al.* (1977) exposed the postincubation suspension of neutrophils to lysostaphin. This muralytic enzyme lysed only the attached bacterial population. Lastly, Besterman *et al.* (1982*b*), after incubation of macrophage monolayers with ^{99m}Tc-labelled zymosan, exposed the cell layer to an excess of unlabelled zymosan to exchange unlabelled for radioactive zymosan reversibly-bound to the cell membrane. When the reversibly-bound compartment was quantified, it was found to comprise a significant and variable (interexperimental) proportion of the total cell-associated particles. These findings underscore the importance of measuring and correcting for the surface-bound compartment when quantifying phagocytosis.

Factors regulating phagocytosis

Surface properties of the particle. The hydrophobicity, charge, and chemical composition of the surface of a particle play an important role in determining the nature of particle–cell interaction. Mudd & Mudd (1924) first postulated that encapsulated bacteria are much less readily ingested by mammalian phagocytes than are unencapsulated variants of the same species because of diminished hydrophobic forces at the surface of the capsule. This hypothesis, extended to include effects of particle charge, has received experimental support from a variety of systems using both bacterial and fungal substrates (van Oss & Gillman, 1972; Stendahl & Edebo, 1972; Stendahl *et al.*, 1973, 1974, 1977*a*; Magnusson *et al.*, 1979; Pesanti, 1979; Kozel *et al.*, 1980). In addition, evidence indicates that hydrophobic forces are involved in the phagocytosis of latex beads and erythrocytes (Walter *et al.*, 1968; van Oss, 1978; Capo *et al.*, 1979), two of the most commonly used phagocytic substrates *in vitro*. Thus, the physicochemical properties of the particle surface are responsible, at least in part, for its fate [for more extensive discussion of this concept see Wilkinson (1976) and van Oss (1978)].

Since the work of Ashwell & Morell (1974) it has become widely recognized that the carbohydrate composition of the particle surface or of an individual glycoprotein plays an important role in endocytic recognition. Galactose units on asialoerythrocytes (Kolb & Kolb-Bachofen, 1978), glucose–galactose units on bacteria (Freimer *et al.*, 1978), mannose/*N*-acetylglucosamine units of glycoproteins and lysosomal glycosidases (Stahl *et al.*, 1978), sialic acid units on sheep erythrocytes (Czop *et al.*, 1978*a*) and mannose/glucosamine units on yeast cell walls (Warr, 1980) are clearly involved in phagocyte recognition and particle attachment both *in vivo* and *in vitro*.

The surface of a particle may be modified by substances in the surrounding milieu which enhance particle–cell interaction. This latter phenomenon is the basis for the concept of opsonization (for reviews see Saba, 1970; Wilkinson, 1976; Silverstein *et al.*, 1977; Walters & Papadimitriou, 1978). Opsonins are serum proteins (e.g. immunoglobulins, complement and fibronectin) that coat the particle and promote attachment to and ingestion by phagocytes (Blumenstock *et al.*, 1978; Gudewicz *et al.*, 1980). The proposed mechanisms by which opsonins enhance particle–cell interaction are of two types: (1) opsonins bind to both particles and to receptors on the surface of the phagocyte, effectively linking the particle to the cell surface (Messner & Jelinck, 1970; Mantovani *et al.*, 1972; Reynolds *et al.*, 1975; Scribner & Fahrney, 1976; Fearon, 1980); and (2) opsonins alter the physicochemical surface properties of the particle [i.e. charge and hydrophobicity; Mudd *et al.* (1934); van Oss & Gilman (1972); van Oss *et al.* (1974); Stendahl *et al.* (1974, 1977*b*); Magnusson *et al.* (1979)]. Regardless of which mechanism is primary, immune opsonization does not make all particles equally susceptible to phagocytosis, since, for example, resident mouse peritoneal macrophages do not ingest complement-coated erythrocytes, but do ingest complement-coated zymosan particles. Thus, surface structures other than complement receptors *per se* govern the ingestion of attached complement-coated particles (Michl & Silverstein, 1978). To complicate matters further, the distribution of immune ligands on the particle surface is a critical factor in determining the

ability of a phagocyte to ingest that particle. Griffin *et al.* (1975, 1976) reported that lymphocytes diffusely coated with antibodies directed against lymphocyte-membrane immunoglobulins were ingested readily by macrophages. Ingestion did not occur, however, when the surface-directed antibodies were first allowed to cap (Taylor *et al.*, 1971). In conclusion, it is difficult to make generalizations concerning which feature of the particle surface governs its interaction with a cell. When immune opsonization is present, its influence usually has been considered predominant, though even this generalization has exceptions (as noted above).

Surface properties of the phagocyte. As with the particle, both the physical and chemical nature of the phagocyte surface membrane are important determinants of its phagocytic function. The physical property given most attention is the hydrophobicity of the cell surface. This parameter is reflected in measurements of the angle a droplet of saline placed on a cell monolayer makes with that monolayer. Using this contact angle technique, van Oss & Gillman (1972) re-established a general physical model for phagocytosis, namely, that a phagocyte will engulf particles whose surface hydrophobicity is greater than its own, but not particles whose surfaces are more hydrophilic than its own. In 1973, Thrasher *et al.* reported that a lymphokine preparation decreased the surface hydrophobicity of cultured macrophages, enhancing their ability to ingest opsonized erythrocytes. Six years later, Vogel & Rosenstreich (1979) and Griffin & Griffin (1979, 1980) reported enhanced immune receptor function imparted to macrophages by lymphokines. Vogel & Rosenstreich (1979) found that macrophages derived from lipopolysaccharide-hyporesponsive C3H/HeJ mice (macrophages known to exhibit a decreased ability to phagocytose immunoglobulin-opsonized sheep red blood cells) demonstrated normal phagocytic capability after exposure to a lymphokine preparation. Similarly, Griffin & Griffin (1979, 1980) found that a lymphokine preparation converted the mouse peritoneal macrophage complement (C3b) receptor system from mediating only particle binding to promoting both the binding and internalization of complement-coated erythrocytes. This lymphokine effect appeared to be specific for complement-mediated phagocytosis, not affecting phagocytosis via the Fc receptor system. More recently, Griffin & Mullinax (1981) suggested that this enhanced C3b receptor function may be due to increased mobility of the receptor within the plane of the plasmalemma (see below).

Alternatively, Vogel *et al.* (1981) reported that the lymphokine treatment of their C3H/HeJ macrophages produced a 60% increase in cellular cyclic AMP and that dibutyryl cyclic AMP and 8-bromo cyclic AMP, as well as other agents known to elevate intracellular cyclic AMP, also corrected the phagocytic defect present in these cells. Such findings are consistent with previous observations that non-phagocytic variants of macrophage-like cell lines (e.g. variants of J774.2) could be rendered capable of ingesting antibody-opsonized erythrocytes by treatment with 8-bromo cyclic AMP (Muschel *et al.*, 1977*a*,*b*). However, it should be noted that increased intracellular cyclic AMP does not significantly affect the phagocytic capability of 'normal' macrophages (e.g. those derived from immune-competent C3H/HeN mice or the parent J774.2 cell line). To what extent the observations of Vogel & Rosenstreich (1979), Vogel *et al.* (1981), Griffin & Griffin (1979, 1980) and Griffin & Mullinax (1981) are related to the change in surface hydrophobicity originally reported by Thrasher *et al.* (1973) remains unclear.

Surface properties of the plasma membrane as a whole may not reflect topographical heterogeneity important for phagocytic function. Griffin & Silverstein (1974) examined whether the response of the plasma membrane to a phagocytic stimulus is localized to the segment of membrane adjacent to the particle initiating the stimulus, or whether the stimulus spreads to a broader area of the cell surface and elicits a general membrane response. Macrophages were incubated with particles that would bind to the membrane, but were not ingested. Then a second particle that the cell would both bind and ingest was added. Ingestion of the second particle did not prompt ingestion of the first particle, even when the site of ingestion of the second particle was adjacent to the segment of membrane to which the first particle was bound. Griffin & Silverstein (1974) concluded that phagocytosis involved a local response of a segment of a cell's surface to signals generated by specific interactions of a particle with the plasma membrane.

This group of workers then asked whether the initial interaction of IgG or C3b ligands on the surface of a particle with receptors on the phagocyte is sufficient to trigger the ingestion of the particle (Griffin *et al.*, 1975). By showing that erythrocytes diffusely coated with IgG or C3b were not ingested when Fc or C3b receptors lying outside the zone of attachment were blocked with anti-macrophage IgG they concluded: (1) that the initial interaction of IgG or C3b ligands on the surface of a particle with receptors on the phagocyte is not sufficient to trigger the automatic ingestion of the particle, and (2) that the phagocytic process requires the continuous apposition of receptors and ligands circumferentially until the particle is fully enclosed within a phagocytic vacuole. This 'zipper' mechanism of phagocytosis views attachment and internalization as a continuum (see below).

Shaw & Griffin (1981) extended the 'zipper'

hypothesis by demonstrating that continuous apposition of cell surface immune receptors and particle-immune ligands is necessary not only to guide pseudopod movement, but also for repeated generation of intracellular phagocytic signals required for particle ingestion. At the same time, continuous apposition of receptor and ligand, though necessary, may not be sufficient to promote particle internalization. Evidence presented by Michl *et al.* (1979*a*,*b*) and Griffin & Mullinax (1981) strongly suggests that lateral mobility of the ligand-bound receptor within the plane of the plasma membrane may be essential for generation of the phagocytic signal.

The phagocytic signal is envisaged as coupling a surface-bound particle to the cytoplasmic machinery of the cell, most likely actomyosin and associated regulatory proteins of the cytoskeleton (Reaven & Axline, 1973; Stossel & Hartwig, 1976; Painter & McIntosh, 1979; Yin *et al.*, 1980; Stendahl *et al.*, 1980). Michl *et al.* (1976*a*,*b*) and Michl & Silverstein (1978) examined whether all surface-bound particles are coupled to the phagocytic machinery in an identical manner, regardless of particle type and the nature of the particle-membrane interaction. They found that: (1) 2-deoxyglucose inhibited Fc and C3b receptor-mediated phagocytosis but not phagocytosis of latex and zymosan particles; (2) attachment of IgG- or C3b-coated erythrocytes to their receptors was not inhibited by 2-deoxyglucose; (3) the effects of 2-deoxyglucose on phagocytosis were unrelated to its capacity to inhibit glucose utilization and ATP generation; and (4) the effects were rapidly reversed by glucose or mannose. Thus, the overall effect of 2-deoxyglucose was to uncouple immune receptors from the phagocytic machinery of the cell while leaving unimpaired other phagocytic pathways within the same membrane segment. The nature of this coupling mechanism remains unknown.

Finally, it should be noted that the phagocytosis of IgG opsonized particles *in vitro* occurs in the presence of whole serum which contains an enormous excess of monomeric IgG. Thus, monomeric IgG is a very poor ligand for the Fc phagocytic receptor and either interaction of IgG with the particle surface (or antigen) must induce a change in the conformation of the Fc domain or the particle serves to crosslink IgG, and it is simultaneous occupation and cross-linking of many receptors that mediates ingestion (Silverstein *et al.*, 1977).

The use of genetic approaches has provided a powerful tool for understanding the mechanism of phagocytosis. Muschel *et al.* (1977*a*) introduced this technique by isolating and cloning mutants of the macrophage-like cell line J774.2. Variants defective in either phagocytosis of IgG-coated erythrocytes (but not latex beads), adenylate cyclase activity, or protein kinase activity were used to investigate the possible role of cyclic AMP in phagocytosis (see above). Results suggest that cyclic AMP plays a regulatory role in Fc-receptor mediated phagocytosis by J774.2 mutants (Muschel *et al.*, 1977*a*,*b*; Rosen *et al.*, 1979), findings consistent with those for macrophages from an immunologically hyporesponsive mouse (Vogel *et al.*, 1981).

Vogel *et al.* (1980) used phagocytic mutants of the unicellular slime mold *Dictyostelium discoideum* to dissect phagocytosis into its individual steps starting with recognition. Initial results identified two mechanisms (sites) for recognition of particle surface properties: (1) a lectin-type receptor mediating binding of particles containing terminal glucose; (2) a 'non-specific' surface mechanism mediating binding via hydrophobic interactions. These findings once again underscore the fact that general physical properties (i.e. hydrophobicity) along with specific receptor-mediated recognition play *bona fide* roles in phagocytosis.

Energy requirements. The binding of particles to cell surfaces generally has been thought of as a passive process, independent of temperature and not requiring metabolic energy. However, there is now evidence in the literature in favour of active binding of unopsonized particles. Michl & Silverstein (1978) found that mouse peritoneal macrophages would not bind unopsonized zymosan at 4°C, though the cells would bind C3b-coated zymosan at this temperature. Warr found that mouse alveolar macrophages would not bind unopsonized *Candida krusei* at 4°C (1980) nor in the presence of metabolic poisons (1979), while Ito *et al.* (1981) reported that mouse peritoneal macrophages showed marked inhibition in their ability to bind albumin-coated paraffin particles with decreasing temperature or in the presence of metabolic inhibitors. Besterman *et al.* (1982*b*) found that binding of ^{99m}Tc-labelled zymosan by monolayers of rabbit alveolar macrophages was inhibited both by NaF and by low temperature (4°C). Although the incubation medium contained 5% serum, the zymosan probably was not functionally opsonized (Czop *et al.*, 1978*b*) since no standard opsonization step was included in particle preparation (e.g. preincubation with whole serum) and since results were the same when heat-inactivated serum was used in the incubation medium. In addition, there are reports of temperature-sensitive binding for opsonized gelatin–latex beads (van de Water III *et al.*, 1981), for opsonized bacteria (Peterson *et al.*, 1977), and for opsonized zymosan (Walter *et al.*, 1980), the temperature sensitivity being independent of an effect on opsonization (Peterson *et al.*, 1977).

In contrast with the controversy surrounding the energetics of particle binding, the literature appears unanimously agreed that particle ingestion is both

energy-requiring and temperature-dependent (Silverstein *et al.*, 1977). In addition, energy-generating pathways of intermediary metabolism are activated during phagocytosis, though membrane–particle interaction (binding) alone is sufficient to trigger this response (Sbarra & Karnovsky, 1959; Cohn & Morse, 1960; Oren *et al.*, 1963).

Divalent cation requirements. Surprisingly, little information is available concerning the divalent cation requirements (i.e. calcium) of phagocytosis, considering the pervasive belief that phagocytic ingestion is a consequence of a mechanism similar to the excitation–contraction coupling which occurs in muscle (Silverstein *et al.*, 1977). Hallgren *et al.* (1978) and Uher *et al.* (1981) have reported a requirement for calcium in the interaction of immunoglobulin-coated red blood cells with the Kupffer cells of perfused liver and with peritoneal macrophages *in vitro*, respectively. Warr (1980) reported that calcium is required for binding of the yeast, *Candida crusei*, by alveolar macrophages. Both Warr (1980) and Uher *et al.* (1981) found that magnesium could not substitute for calcium. Stossel (1973) found that calcium increased the rate of uptake of bovine serum albumin-coated paraffin oil particles by leukocytes, while Ito *et al.* (1981) found that calcium was essential for the attachment of this same particle-type to peritoneal macrophages, but not for particle ingestion. Furthermore, both Stossel (1973) and Ito *et al.* (1981) reported that other divalent cations could replace calcium.

Phagocytic capacity. Phagocytes are known to have a maximal phagocytic capacity (Michell *et al.*, 1969; Werb & Cohn, 1972; Leijh *et al.*, 1979); that is, given a particulate load, they will engulf a finite quantity and then enter a period during which they are phagocytically inactive. Two explanations have been proposed for the appearance of this refractive period: (1) binding sites specific for that particle-ligand have been internalized and are no longer available for particle interaction (Schmidt & Douglas, 1972); (2) there is a maximal level of membrane that can be internalized before phagocytosis must cease in order to allow membrane regeneration (Werb & Cohn, 1972). In support of the former hypothesis, the work of Petty *et al.* (1980) demonstrated that Fc receptor-dependent phagocytosis of IgG-coated liposomes resulted in the loss of Fc-, but not C3b, surface receptor activity. In contrast, Besterman *et al.* (1982*b*) showed that surface-binding of radiolabelled zymosan remained at its maximal value when internalization ceased. Hence, Besterman *et al.* (1982*b*) concluded that a reduced binding capacity could not account for the limited phagocytic capacity. Concerning the latter hypothesis, Petty *et al.* (1981) calculated that during antibody-dependent phagocytosis of IgG-coated liposomes, a surface membrane area equivalent to that of the entire plasmalemma is internalized, yet the measured cell surface area decreased by only 25%. These investigators postulated that during antibody-dependent phagocytosis an internal membrane reservoir is made available to the cell surface.

Clearly, additional research is required to determine which factors act to limit the phagocytic capacity of a cell. It is worth noting that although a single unifying hypothesis, applicable to all systems, is typically sought, the realization that such might not be the case (e.g. that phagocytosis of opsonized and unopsonized particles may not be limited by the same mechanism) may serve to clarify what now appear as contradictions in the literature.

Phagocytosis and plasma membrane dynamics

The fluid mosaic model of the plasma membrane (Singer & Nicolson, 1972; Rothman & Lenard, 1977) depicts the cell membrane as a two-dimensional fluid bilayer into which are intercalated the membrane proteins. Specific membrane proteins are, or can become, attached to cytoplasmic cytoskeletal and contractile proteins (Steck, 1974; Nicolson, 1976), such interactions possibly serving to anchor those membrane proteins in specialized membrane domains, or to direct restricted patterns of lateral mobility within the plane of the membrane.

Of central importance is whether phagocytosed membrane is a representative or selected sample of the plasmalemma from which it arises. This question was first raised by the observations of Tsan & Berlin (1971), who found that the phagocytosis of latex beads by neutrophils and macrophages did not reduce the ability of these cells to transport certain purines and amino acids. Neither the K_m nor the $V_{max.}$ of the surface-transport sites was altered, even though an estimated 30–50% of the cell surface had been interiorized. Tsan & Berlin (1971) concluded that transport sites in the plasma membrane were excluded from the forming phagocytic vacuole. Conversely, Oliver *et al.* (1974) found that in resting polymorphonuclear leukocytes, concanavalin A receptors were distributed at random over the plasmalemma. However, following phagocytosis, the number of concanavalin A binding sites per unit membrane fell appreciably. This decrease was interpreted as indicating that concanavalin A receptors were selectively removed during phagocytosis. Since the concanavalin A receptors were present initially in a random distribution, the most likely explanation seemed to be that there was an induced movement of receptors into the patch of membrane that composed the forming phagosome.

In support of the selection hypothesis are at least two other recent studies. Cramer *et al.* (1980) examined the ultrastructural localization of 5′-nucleotidase (a plasmalemma enzyme marker) in

polymorphonuclear leukocytes at rest and during phagocytosis of heat-killed bacteria or latex microbeads. They concluded that 5′-nucleotidase is absent from phagosome membranes due to movement and redistribution of the enzyme during phagocytosis. Nowakowski & Bianco (1980) iodinated macrophage surface membrane, fed these cells immunoglobulin or complement-opsonized erythrocytes, isolated the phagolysosomes and compared their labelled polypeptide profile with that of the iodinated surface polypeptides. They concluded that interiorization of iodinated macrophage membrane during immune phagocytosis is selective and substrate (nature of the opsonin)-specific.

In apparent conflict with the 'selection hypothesis' is the work of Muller *et al.* (1980*a*,*b*) who examined the iodination of polypeptides of the phagolysosomal membrane using lactoperoxidase covalently coupled to carboxylated latex beads. They concluded that membrane proteins accessible to lactoperoxidase-catalysed iodination on the luminal surface of the phagolysosome and on the external face of the plasmalemma were similar, if not identical.

Insight into the apparently contradictory nature of these reports may be gained from the observations of Petty *et al.* (1980). As already mentioned, these workers reported that Fc receptor-dependent phagocytosis of IgG-coated liposomes (containing 1–2% phospholipid hapten) resulted in a loss of Fc-, but not C3b-, surface receptor activity from the murine macrophage cell line RAW264. In contrast, the phagocytosis of unmodified latex beads resulted in the loss of both Fc- and C3b-surface receptor activity. These findings illustrate that the nature of the substrate particle is likely to determine its mode of interaction with the cell (see the section on Surface properties of the particle) and, in turn, may determine the composition of the resulting phagosome membrane.

Another approach has been to study the interrelationship of two endocytic processes occurring simultaneously, phagocytosis and fluid-phase pinocytosis. Berger & Karnovsky (1966) found that phagocytosis of polystyrene spherules increased the accumulation of intracellular [^{14}C]inulin by guinea pig polymorphonuclear leukocytes, while Bowers (1977) found that the concomitant phagocytosis of latex beads decreased the rate of [^{3}H]inulin pinocytosis in *Acanthamoeba*. Most recently, Besterman *et al.* (1982*b*) found that fluid-phase pinocytosis by rabbit alveolar macrophages was unaffected by concomitant phagocytosis of zymosan or latex beads. Conclusions concerning the interdependence of phagocytosis and fluid-phase pinocytosis must be reserved until it is known whether cell-type and particle-type differences are responsible for the contradictory nature of these findings.

Pinocytosis

Measurement of fluid-phase pinocytosis

A host of markers have in the past been used to study fluid-phase pinocytosis (for reviews, see Silverstein *et al.*, 1977 and Pratten *et al.*, 1980). Electron-opaque markers proved useful for intracellular localization, but unless the markers were also enzymically active or radioactive they were difficult to quantify. Such bifunctional probes included colloidal gold (^{198}Au, a gamma emitter) and horseradish peroxidase (radioiodinated or uniodinated; Steinman & Cohn, 1972). However, it has been demonstrated recently that horseradish peroxidase (Kaplan & Nielsen, 1978), ^{198}Au and ^{125}I-labelled albumin (Pratten *et al.*, 1977) adsorb to the cell membrane and enter the cell at least partly via adsorptive pinocytosis. Thus, for quantification of fluid-phase pinocytosis without microscopy, radiolabelled sucrose, inulin, and polyvinylpyrrolidone have been found most suitable. (For review on the use of radiolabelled sucrose, see Besterman *et al.*, 1981; for inulin see Bowers & Olszewski, 1972; and for polyvinylpyrrolidone see Williams *et al.*, 1975 and Roberts *et al.*, 1977.)

The intracellular accumulation of a fluid-phase marker is characterized by a rate which is directly proportional to the concentration of the marker in the medium. However, the literature is replete with apparent contradictions concerning the kinetics of accumulation of fluid-phase markers with time, and the problem requires final resolution both with regard to its implication for mechanism and its importance for quantification of the process. Some reports describe uptake as linear for up to 6h (Becker & Ashwood-Smith, 1973; Pratten *et al.*, 1977; Roberts *et al.*, 1977; Berlin *et al.*, 1978) while others report linear accumulation of one marker and nonlinearity of another in the same cell type (Roberts *et al.*, 1977; Davies & Ross, 1978). Recently, Besterman *et al.* (1981) investigated the uptake and fate of pinocytosed fluid in monolayers of pulmonary alveolar macrophages and foetal lung fibroblasts by using [^{14}C]sucrose. Initial experiments revealed that cellular accumulation of chromatographically-repurified [^{14}C]sucrose was not linear with incubation time. Deviation from linearity was shown to be due to constant exocytosis of accumulating marker (Fig. 1). Computer simulation showed that the rapid kinetics of exocytosis exactly accounted for deviation from linearity of [^{14}C]-sucrose uptake and allowed calculation of actual pinocytic rates, found to be nearly 50% greater than the apparent initial rate measured over the first 10min. Thus, failure to measure carefully events early after introduction of a pinocytic marker may cause significant interpretative errors both in terms of absolute endocytic rates and in terms of com-

parisons where flux is altered. In addition, these results demonstrated that the use of prolonged washing procedures in uptake experiments such as employed by Davies & Ross (1978) and Besterman *et al.* (1982*b*) may result in uptake kinetics which falsely appear linear.

Factors regulating fluid-phase pinocytosis

In contrast to the information known about the mechanism of phagocytosis, the control of fluid-phase pinocytosis is very poorly understood. Generalities concerning extracellular factors that alter the rate of fluid-phase pinocytosis should be viewed with caution because the magnitude and direction of the observed effects are variable and apparently cell-type dependent. Serum (Cohn & Benson, 1965; Kaplan & Nielson, 1978), ATP (Cohn & Parks, 1967*b*), fetuin (Cohn & Parks, 1967*a*; Kaplan & Nielsen, 1978), histones (Ryser, 1968; Kaplan & Nielsen, 1978), glucose (Becker & Ashwood-Smith, 1973), concanavalin A (Edelson & Cohn, 1974), epidermal growth factor (Haigler *et al.*, 1979), phorbol myristate acetate (Phaire-Washington *et al.*, 1980), the charge on the marker molecule (Davies *et al.*, 1980), the presence of other polyamines or polycations (Ryser, 1968; Drummond *et al.*, 1980), the developmental history of the cell (Edelson *et al.*, 1975; Phaire-Washington *et al.*, 1980), the phase in the cell cycle (Berlin *et al.*, 1978; Berlin & Oliver, 1980; Quintart *et al.*, 1979) and cell density in culture (Steinman *et al.*, 1974) have been pointed to as regulators of fluid-phase pinocytosis.

Interestingly, from a mechanistic point of view, constitutive pinocytosis appears not to require extracellular divalent cations (Besterman *et al.*, 1981). Similarly, calcium also does not appear to be required for continuous secretion of such products as immunoglobulins (Tartakoff & Vassalli, 1977) collagen (Tartakoff & Vassalli, 1978) and secretory proteins (Tartakoff & Vassalli, 1978). At the same time, the divalent cation is required for those cases where secretion occurs only under appropriate stimuli, as for the pancreas (Hokin, 1966; Curry *et al.*, 1968), adrenal medulla (Douglas, 1966), and neurohypophysis (Douglas & Poisner, 1964).

In contrast to constitutive pinocytosis of mammalian cells, pinocytosis in many protozoans is an induced endocytic event and has provided a model process for studying membrane–cytoplasmic signal transduction. Induction of pinocytosis in amoeboid cells is accompanied by displacement of cell surface calcium (Prusch & Hannafin, 1979) with an increase in plasmalemmal ionic permeability (Brandt & Freeman, 1967), including a calcium influx (Prusch & Dunham, 1972). Increased cytosolic calcium may provide the signal for arrangement and contraction of a surface membrane-associated contractile filament network, resulting in surface invagination, pinocytic channel formation, and vesiculation (Klein & Stockem, 1979; Prusch, 1980; Taylor *et al.*, 1980*a*,*b*).

Fluid-phase pinocytosis and plasma membrane dynamics

Historically, endocytosis and exocytosis have been approached separately by two schools of thought, each school virtually ignoring the fact that, though the cell type being investigated was obviously specialized to perform one of these functions, each function might be coupled to its vectoral mate of opposite direction. Though originally predicted by Palade in 1959, it was not until the membrane revolution in the late 1960's and early 1970's that those studying specialized secretory cells demonstrated that these cells exhibited an enhanced rate of fluid-phase pinocytosis for minutes to hours following a burst of secretory activity (neurons, Heuser & Reese, 1973; exocrine pancreatic cell, Geuze & Kramer, 1974; endocrine pancreatic cells, Orci *et al.*, 1973). Conversely, it was not until even more recently that those investigators primarily concerned with the endocytic pathway succeeded in demonstrating that exocytosis was a concomitant process in these cells (Schneider *et al.*, 1979; Muller *et al.*, 1980*b*). It was the realization that cells must recycle membrane as a homeostatic and conservatory measure that led to the heretofore mentioned findings that a given cell type is capable of shuttling membranous vesicles both to and from the plasmalemma, by a process which at the same time also serves to transport other important molecules into or out of the cell. Thus, endocytosis and exocytosis represent opposing limbs of an intracellular transport system that probably operates in all eukaryotes.

The area of membrane interiorized during endocytosis can be estimated by stereology, a statistical method for obtaining three-dimensional information from two-dimensional electron micrographs. Steinman *et al.* (1976), using this approach, found the surface area of macrophages and L-cells actually to be three times the area of smooth spheres of equivalent volume. This comes as no surprise considering the active 'ruffled' cell membrane these cells are known to possess. Using horseradish peroxidase as a marker, these investigators then examined the rates of volume and surface internalization due to fluid-phase pinocytosis. They found that macrophages pinocytose 25% of their cell volume and 186% of their cell surface area each hour. Yet, throughout the 3 h period studied, total cell volume and area remained constant, and both the pinocytic vesicle and secondary lysosomal compartment occupied a constant volume and area, equivalent to 2–3% and 15–20% of the total cell volume and surface area, respectively. Qualitatively

similar results were found for L-cells. Thus, the volume and surface area of incoming pinocytic vesicles is ten times the steady-state dimensions of both the pinocytic vesicles and lysosomal compartment. These results led Steinman *et al.* (1976) to propose that recycling of internalized membrane back to the cell surface and rapid egress of pinocytic fluid must continuously occur (Fig. 1).

Besterman *et al.* (1981) tested the latter prediction by closely examining the fate of the pinocytosed fluid-phase marker [^{14}C]sucrose. A detailed analysis of the kinetics of exocytosis was undertaken by preloading cells with [^{14}C]sucrose for varying lengths of time and then monitoring the appearance of radioactivity into isotope-free medium. Results indicated that modelling the process of fluid-phase pinocytosis and subsequent exocytosis required at least two intracellular compartments in series, one compartment being of small size and turning over very rapidly ($t_{\frac{1}{2}} = 5$ min in macrophages, 6–8 min in fibroblasts) and the other compartment being apparently larger in size and turning over very slowly ($t_{\frac{1}{2}} = 180$ min in macrophages and 430–620 min in fibroblasts). Moreover, the sizes of the compartments and magnitude of the intercompartment fluxes were such that most of the fluid internalized in pinocytic vesicles was rapidly returned to the extracellular space via exocytosis (Fig. 1). This result provided direct experimental evidence for a process previously thought necessary based solely on morphological and theoretical considerations.

The question again arises as to whether the membrane which is internalized, in this case during pinocytosis, is a representative or selected sample of the plasma membrane. Taylor *et al.* (1971) found that following incubation with anti-immunoglobulins, the surface of B lymphocytes is cleared of its immunoglobulin binding sites by pinocytosis, but binding of antibodies to other surface components (e.g. histocompatibility antigens) is not altered. In contrast, Mellman *et al.* (1980), using a novel technique which appeared to radioiodinate specifically pinocytic vesicles, concluded that the pinocytic vesicle membrane is formed from a representative sample of plasma membrane polypeptide components.

There appear to be at least two explanations for the inability to resolve unequivocally this question. Firstly, conflicting results may partly or entirely be due to differences in methodology. Secondly, it must be remembered that there are at least two distinctly different mechanisms operating, fluid-phase and receptor-mediated pinocytosis. The importance of this point is illustrated by the fact that ligands bound to cell-surface receptors can redistribute prior to endocytosis. This redistribution, and not the internalization step *per se*, may be the selective process; and the internalized membrane may otherwise be identical with the plasma membrane from which it was derived.

Speculation on the functions of fluid-phase pinocytosis

Since a recycling process now appears a likely component of fluid-phase pinocytosis, the possibility exists for intracellular selectivity and modification of pinocytosed solutes. Though fluid-phase pinocytosis in protozoans shows characteristics different from those of mammalian cells, it is worth noting that *Amoeba proteus* demonstrates selectivity in its extrusion of pinocytically-ingested material. Specifically, Prusch (1981) reported that *Amoeba proteus* appears to eliminate selectively potentially harmful pinocytosed material (i.e. Alcian Blue) through a variety of extrusion mechanisms. If such mechanisms operate in mammalian cells remains to be examined.

Degradation of solute macromolecules appears a likely form of intracellular modification, and probably occurs at the level of the lysosome (Ehrenreich & Cohn, 1967; Dingle *et al.*, 1973; Goldstein *et al.*, 1975; Poole *et al.*, 1980). For example, it has been speculated often that constitutive pinocytosis of serum proteins subserves the nutritional role of providing a potential source of amino acids for protein synthesis *de novo*, although early experiments concluded otherwise (Eagle & Piez, 1960; Ryser *et al.*, 1962). However, Hammer & Rannels (1981) have now reported that in the absence of extracellular amino acids, protein synthesis by rabbit alveolar macrophages decreased by 30% but the presence of exogenous protein (2% bovine serum albumin) could restore the synthetic rate to normal. The work of Low (see Rannels *et al.*, 1982) also suggested that exogenous protein (e.g. 10% foetal bovine serum) could be utilized as a source of amino acids for protein synthesis by guinea pig alveolar macrophages *in vitro*. Furthermore, Besterman *et al.* (1982*a*) have provided evidence that the pinosome–lysosome pathway is sensitive to the availability of extracellular free amino acids, making likely the possibility that pinocytosis coupled to intracellular degradation of exogenous proteins could provide a significant source of amino acids for protein synthesis, especially under conditions of amino acid deprivation.

A popular hypothesis is that pinocytosis serves as a pathway for continual turnover of plasma membrane lipid and/or protein. Bretscher (1976) proposed a directed lipid flow model to explain the phenomenon of capping of crosslinked surface antigens on fibroblasts and other motile cells. Necessary for that model is the existence of a molecular filter, an endocytic mechanism for the selective internalization of plasma membrane lipid with the exclusion of most plasma membrane

proteins. The finding that two surface proteins of mouse 3T3 fibroblasts are excluded from coated pits has been used to argue that these endocytic structures may serve as the hypothetical molecular filter (Bretscher *et al.*, 1980). This model in no way excludes the possibility that constitutive pinocytosis (probably not mediated by coated pits) contributes to continual plasma membrane turnover. With regard to turnover of plasmalemmal protein, constitutive pinocytic rates indicate that 54–186% of the cell surface area is internalized per hour (fibroblasts–macrophages; Steinman *et al.*, 1976) a rate which far exceeds the $2\% \cdot h^{-1}$ turnover rate for membrane proteins (Warren & Glick, 1968; Hubbard & Cohn, 1975). Thus: (1) constitutive pinocytosis is not required solely as a mechanism for plasmalemma protein turnover, and (2) recycling of membrane proteins is likely to be a consequence of a continuous endocytic–exocytic shuttle (Schneider *et al.*, 1979; Muller *et al.*, 1980*b*).

Receptor-mediated endocytosis

Receptor-mediated endocytosis involves the binding of the ligand to receptors on the plasma membrane (usually diffusely distributed), followed by clustering of the ligand–receptor complexes in coated pits. These specialized regions of the plasma membrane are coated on their cytoplasmic surface with a lattice-work of the protein clathrin (Pearse, 1975, 1976, 1978). It has been generally assumed that the coated pits pinch off to form coated vesicles and that the coated vesicles usually deliver the ligand to lysosomes or other intracellular compartments with the recycling of the clathrin and receptors back to the plasmalemma (for reviews, see Goldstein *et al.*, 1979; Ockleford & Whyte, 1980; Pearse, 1980; Pastan & Willingham, 1981). This scenario was suggested: (1) by electron micrographs showing coated membrane regions (pits) and coated vesicles (Roth & Porter, 1964; Friend & Farquhar, 1967; Anderson *et al.*, 1977*a*); (2) by the isolation of closed coated vesicles from homogenized cells (Kanaseki & Kadota, 1969; Pearse, 1975, 1976; Woodward & Roth, 1978; Keen *et al.*, 1979); and (3) by kinetic studies which demonstrated that when synthesis of new receptors was blocked by cycloheximide, receptor-mediated endocytosis was unaffected (Goldstein *et al.*, 1976; Anderson *et al.*, 1977*b*).

Recent ultrastructural and biochemical evidence raises questions concerning the formation of coated vesicles and the need for clathrin to recycle. Specifically, ultrastructural analysis of fibroblasts by Willingham & Pastan (1980) suggest that the clathrin coat remains permanently attached to the plasma membrane. They postulated that receptor-bound ligands, once clustered in coated pits, are transferred to uncoated vesicles; these vesicles are formed either as an invagination from adjacent uncoated plasmalemma or via an opening in the clathrin coat. Willingham & Pastan (1980) named these uncoated vesicles 'receptosomes'. Wall *et al.* (1980) described a pathway involving structures similar to 'receptosomes' for receptor-mediated endocytosis of asialoglycoproteins by hepatocytes. Willingham *et al.* (1981) reported that immunocytochemical localization of clathrin with anti-clathrin antibodies detected clathrin on the coated regions of both plasma membrane and GERL, but no soluble clathrin was found in either the cytosol or in association with other organelles. Wehland *et al.* (1981) provided ultrastructural evidence that although microinjected anticlathrin antibodies gained access to all extramembranous cytosolic compartments (and could bind clathrin microinjected into the cytoplasm), the anticlathrin antibody neither induced aggregates of endogenous clathrin, nor affected ongoing receptor-mediated endocytosis, but only labelled pre-existing coated pits.

Taken together, these results suggest a modified model of receptor-mediated endocytosis, a model in which receptor-bound ligand aggregates in coated pits and the ligand–receptor complexes are transferred to uncoated vesicles ('receptosomes'). The 'empty' coated pit remains in continuity with the plasma membrane, precluding the formation of coated vesicles and, hence, the necessity for clathrin recycling (Fig. 1).

Conclusion

Considerable progress has been made toward defining the mechanisms governing endocytosis. Appreciation for the general physicochemical properties involved in particle–cell interaction as well as the many specific ligand–receptor mechanisms has led to modelling phagocytosis with the recognition that each mode of phagocytosis may operate by a different set of rules and regulators. The fact that fluid-phase and receptor-mediated pinocytosis appear to be constitutive processes involved with the constant flux of the plasmalemma and intracellular membrane compartments has emphasized the central role of plasma membrane dynamics in endocytic function. Technical innovation in assaying discrete events in the endocytic process on a quantitative basis, as well as the introduction of mutant cell lines, has facilitated many of these advances.

For the future, expanded use of genetic mutants in conjunction with the development of heretofore non-traditional techniques to the field (e.g. single-cell electrophysiology) will be the key to further understanding of the steps of phagocytosis as well as the

complex network of intracellular endocytic pathways.

Though, to date, research into endocytosis has focused on but a few cell types, it is likely that all forms of this cell function operate in most eukaryotic cells. In this regard, although many putative regulatory substances have been identified *in vitro*, establishing their relevance *in vivo* looms as a major task ahead.

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